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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Craig and Colyer et al.
Serial No.:	09/511,776
Filed:	February 24, 2000
Entitled:	METHODS AND COMPOSITIONS USING PROTEIN BINDING PARTNERS

Examiner: Gabel

Art Unit: 1641

Atty. Docket No.: 10069/11700 {formerly 4256/86197}

Box: Non-fee Amendment
Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 CFR 1.132 BY DEREK N. WOOLFSON

I declare:

1. I, Derek N. Woolfson hold a Ph.D. degree in Chemistry from the University of Cambridge. I received my Ph.D. degree in 1992. My current position is Senior Lecturer in Biochemistry at the University of Sussex. I have held this position since 1999. Previously, I held the position of Lecturer in Biochemistry at the University of Sussex from 1996. I am not an inventor of the above-referenced patent application.

2. I have read the Office Action dated June 7, 2001, filed in the above-referenced patent application and understand that the Examiner has rejected claims 1, 3 and 7-11 for alleged lack of novelty and/or for obviousness. The Examiner has suggested that the covalently bound fluorescent indicators and methods of using these indicators to determine the concentration of an analyte, as taught in U.S. 5,998,204 (Tsien et al.) anticipate claims 1, 3 and 7-11 of the instant

application.

3. I have performed scientific research in the field of protein structure, protein-protein interactions and the regulation thereof, and methods of determining protein conformations. It is my opinion that one of skill in the art would consider me to be an expert in the fields of protein structure, protein interaction, and methods of determining protein conformations.

4. The use of a first and a second binding partner that are not coupled to a protein, as claimed in the above referenced patent application, enables the use a variety of different chemical fluorophores for each binding partner, and allows different reaction conditions to be used for the labeling of the first binding partner and the second binding partner. The covalently associated fluorescent indicators of U.S. 5,998,204 can not be labeled in separate reactions.

5. A method for determining the conformational state of a protein that utilizes a first and second binding binding partner that are not covalently coupled to the protein can be carried out using only one fluorescent label, for example using the fluorescence polarisation method described below. In contrast, if the claimed method of the conformational state of a protein were performed with the covalently coupled fluorescent indicators of Tsien et al., U.S. 5,998,204, each of the coupled fluorescent indicator molecules would have to be labeled with a fluorescent label.

6. It is my view as an expert in this field that the claimed method of determining the conformational state of a protein that utilizes a protein and first and second binding partners that are not covalently associated with the protein, provides for an assay that is (1) potentially faster; (2) more efficient, because the components of the reporter complex are independent and the conformational change will result in complete disassembly, whereas with a linked complex the reporter subunits will remain proximal; and (3) such a system would be easier to develop and optimise than one using the covalently associated fluorescent indicators of Tsien et al., because the claimed method does not require a linker moiety connecting the protein and binding partners.

To efficiently perform the method of Tsien et al., the length of the linker moiety between the two fluorescent indicators must be empirically defined and then a molecule comprising the two fluorescent indicators and the linker of the desired length, must be expressed, purified and then tested. One of skill in the art would accept that the claimed method does not require such a testing step and could therefore be carried out more rapidly since there is no need to first optimise the experimental parameters of linker length.

7. The method of Tsien et al. requires the use of a large protein molecule comprising two covalently attached fluorescent indicator molecules and a binding protein moiety. It is my belief that it is more advantageous to perform the claimed method with molecules that are not covalently attached than the Tsien molecules (for example, the non-covalently associated protein and binding partner(s) of the claimed method) because of the following:

a) With multiple, covalently linked protein modules there is increased likelihood of mis-assembly; there are several examples of misfolded oligomers in which unnatural structures are assembled from different polypeptide chains brought "unnaturally" into close proximity, this is known as "domain-swapping" (see Liu *et al.* 2001, Nature Structural Biology **8** p. 211). Isolated protein modules, on the other hand, are most likely to fold correctly and independently prior to complex assembly, therefore, allowing for detection of the conformational change being assayed.

b) In addition, with covalently linked proteins the linker regions may be susceptible to degradation through proteolysis, which could lead to dis-assembly of the reporter complex. To avoid, this many variant linker regions may have to be made and those least prone to proteolysis selected. Using isolated, independently folded protein units avoids this potential problem.

c) Finally, the claimed invention teaches a method of detecting a conformational change that can utilize the unaltered, native protein, thereby increasing the probability of the protein to fold into the appropriate conformation, whereas if the claimed method were carried out with the covalently associated fluorescent indicators of Tsien et al., the native protein could not be used.

8. The claimed method for determining the conformational state of a protein that utilizes a first and second binding partner wherein the first and second binding partner are not covalently

coupled to the protein, can be designed such that the signal that is generated is optimized by altering the ratio of first binding partner to the second binding partner. For instance it may be advantageous to perform the claimed method in the presence of an excess of a first binding partner to increase the detection sensitivity. In certain embodiments, the claimed method may be carried out by titrating in the first binding partner such that the generated signal is derived from the second binding partner. Since the ratio of the covalently attached fluorescent indicators taught in U.S. 5,998,204 are fixed at 1:1, such optimization and titration are not possible if the claimed method is carried out with a covalently associated protein-binding partner pair.

9. The molecule comprising the coupled fluorescent indicators of the Tsien et al. reference is a single polypeptide. To produce different forms of the Tsien molecules, wherein the molecule comprises different combinations of coupled associated fluorescent indicators, a new construct must be constructed, expressed and purified from some type of cellular expression system for each molecule. To perform the methods of the claimed invention, large batches of the protein, large batches of the first binding partner and large batches of the second binding partner can be prepared. An aliquot of the first binding partner and the second binding partner pool can be removed and labeled by independently, covalently attaching the appropriate chemical label under optimum labeling conditions. Thus, numerous variations of the method of the claimed invention can be performed with only a single batch of first binding partner and a single batch of second binding partner. The method of the claimed invention can also be carried out using a first binding partner and a second binding partner comprising a label that produces a signal that is at least detectable and preferably optimal, for any available detection system. Thus, the method of the claimed invention is extremely versatile.

10. A method for determining the conformational state of a protein that utilizes a protein and a first and second binding partner, wherein the protein is not covalently associated with either the first or second binding partner enables the use of a variety of detection methods other than FRET. The detection methods discussed below (surface plasmon resonance, fluorescence

polarization, and immobilized assays for detecting protease activity) would not work with a protein coupled to at least one of a first and second binding partner.

Surface plasmon resonance (SPR) is a detection method wherein one entity is bound to a chip surface and a second entity is passed over the chip. Binding is detected by a change in the surface plasmon resonance. This method does not require any fluorescent label, but does require two separate, unconnected, entities (for example a non-associated protein and first and/or second binding partner(s)) to achieve a change in molecular mass that occurs upon binding.

Fluorescence polarization (FP) is a detection method wherein a first and/or second binding partner is a short peptide and is labeled with a low molecular weight fluorescent label such as fluorescein. Upon binding to the protein the rotation rate in solution of the first and/or second binding partner is decreased and can be measured by a change in the FP index. It is required that the entities that are being assayed for binding be unattached prior to binding. In general fluorescent molecules (that is, the total combination of label and polypeptide) that are greater than 16kDa are too large to be used for FP.

Immobilized assays (for example, wherein one of the first or second binding partners are bound to a solid substrate) for determining the conformation of a protein have the advantage of allowing an unbound molecule to be washed away, thus reducing background signal produced by unbound fluorophore. An immobilized assay can not be performed with a protein that is covalently coupled to a first and/or second binding domain pair.

I hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

24-08-07.

Date

Derek N. Woolfson